Diabetologia (1996) 39: 1091–1098

Diabetologia

Diagnostic sensitivity of immunodominant epitopes of glutamic acid decarboxylase (GAD65) autoantibodies in childhood IDDM

A. Falorni[1], M. Ackefors[1], C. Carlberg[1], T. Daniels[2], B. Persson[3], J. Robertson[4], Å. Lernmark[1,2]

[1]Department of Molecular Medicine, Karolinska Institute, Stockholm, Sweden
[2]Department of Medicine, University of Washington, Seattle, Washington, USA
[3]Department of Women and Child Health, Karolinska Institute, Stockholm, Sweden
[4]Synectics Biotechnology, Stockholm, Sweden

Summary The prevalence and titre of epitope-specific autoantibodies to glutamic acid decarboxylase (GAD65) in 155 insulin-dependent diabetic (IDDM) and 9 GAD65 antibody (Ab)-positive healthy children were determined using four GAD65/67 chimaeric molecules which discriminate among the N-terminal (N), middle (M) and C-terminal (C) epitopes of GAD65. Radioligand binding assays for IgG Ab used immunoprecipitation of in vitro translated ^{35}S-GAD. We found autoantibodies to GAD65 in 116 of 155 (75 %), to GAD67 in 19 of 155 (12 %) ($p < 0.0001$) and to the GAD65-N-67 chimaera in 25 of 155 (16 %) ($p < 0.0001$) IDDM sera. GAD67Ab were found almost exclusively (17 of 19, 89 %) in GAD65Ab-positive sera and the levels of GAD67Ab correlated with those of GAD65Ab ($r^2 = 0.5913$; $p = 0.009$). GAD65Ab directed to GAD65-M were found in 104 of 155 (67 %), to GAD65-C in 104 of 155 (67 %) and to GAD65-M + C in 116 of 155 (75 %) of IDDM sera, and indicated reactivity to at least two distinct epitopes. Among the nine GAD65Ab-positive healthy children, two (22 %) were also positive with GAD67, nine (100 %) with GAD65-M + C, seven (78 %) with GAD65-M, eight (89 %) with GAD65-C and two (22 %) with GAD65-N-67. Titres of GAD65Ab ($p = 0.007$), GAD65-C-Ab ($p = 0.002$) and GAD65-C + M-Ab ($p = 0.003$), but not of GAD65-M-Ab ($p = 0.101$) were significantly higher in IDDM than in healthy children. We conclude that GAD65Ab in IDDM and healthy children are directed to middle and C-terminal epitopes, and propose that levels of antibodies specifically directed to the carboxy-terminal end of GAD65 may distinguish IDDM from healthy children. [Diabetologia (1996) 39: 1091–1098]

Keywords Insulin-dependent diabetes mellitus, autoimmunity, autoantibodies, radioimmunoassay, recombinant protein.

The M_r 65 000 isoform of glutamic acid decarboxylase (GAD65) [1] is the single isoform expressed in human islets and represents the 64 K islet autoantigen originally detected in sera from patients with insulin-dependent diabetes mellitus (IDDM) [2–4]. GAD65 autoantibodies (GAD65Ab) occur in 70–75 % of recent-onset IDDM patients in both Caucasian [5–9] and other ethnic groups [10, 11]. In contrast, autoantibodies to the M_r 67 000 isoform of GAD (GAD67) occur in only 15–20 % of IDDM patients [8, 9] and, in most instances, epitopes are shared between the two GAD isoforms [11]. The presence of GAD65Ab in healthy first-degree relatives of IDDM patients may be predictive for IDDM [12] especially in individuals positive for other IDDM-related markers such as islet cell antibodies (ICA) or insulin autoantibodies [13]. However, a limitation in the use of humoral immune markers to predict IDDM is the inadequate sensitivity and specificity of currently available assays in relation to the low prevalence of IDDM in the general population [14, 15]. Accordingly, the

Received: 17 January 1996 and in revised form: 18 March 1996

Corresponding author: Dr. A. Falorni, Laboratory for Molecular Immunology, Department of Molecular Medicine, Karolinska Hospital, M3:00, S-171 76 Stockholm, Sweden

Abbreviations: Ab, Antibodies; GAD, glutamic acid decarboxylase; ICA islet cell antibodies; IDDM, insulin-dependent diabetes mellitus; JDF, Juvenile Diabetes Foundation; PC-2, proinsulin-converting enzyme 2; TCA, trichloroacetic acid; M, middle terminal epitope; N, N-terminal epitope; C, C-terminal epitope.

identification of IDDM-specific GAD65Ab epitopes and the titration of epitope-specific GAD65Ab may increase the positive predictive value of this marker for IDDM.

In IDDM sera, GAD65Ab appear to be predominantly directed to epitopes located to the middle (M) and carboxy-terminal (C) regions of GAD65 [16, 17]. In contrast, GAD65Ab associated with other autoimmune diseases, such as stiff-man syndrome or the autoimmune polyendocrine syndrome type I, are directed to a different and probably broader spectra of epitopes [18–20].

The frequency of GAD65Ab in healthy individuals is low, at approximately 2 % or less [8, 9, 11] and no information is currently available on their epitopes. Epitope analysis of a large number of recent-onset IDDM patients is needed to determine disease specificity and levels of IDDM-associated epitope-specific GAD65Ab. Development of different epitope-specific autoantibodies among IDDM patients may indicate different pathways of pathogenesis, while the conservation of autoantibody epitopes may suggest common mechanisms in the formation of GAD65Ab in the pathogenesis of IDDM.

The aim of the present study was to use hybrid molecules generated by substitution of regions of GAD65 with homologous regions of GAD67 to determine the diagnostic sensitivity of epitope-specific GAD65 autoantibodies for IDDM in a large group of Swedish recent-onset childhood IDDM patients and a group of GAD65Ab-positive healthy schoolchildren.

Subjects, materials and methods

Human serum samples. Serum samples from 155 Swedish recent-onset IDDM patients (male/female ratio: 84/71; age 1–18 years) and from 84 GAD65Ab-negative healthy children (male/female ratio: 37/47, age 8–18 years) previously used to develop a semi-automated radiobinding assay for GAD65Ab [21], were used in this study. The IDDM children were consecutively diagnosed during the period 1988–1992 at St. Görans Hospital, Stockholm, Sweden. GAD65Ab were detected in 116 of 155 (75 %) IDDM patients and 1 of 85 (1.2 %) healthy individuals [21]. To analyse the GAD65Ab epitopes among healthy individuals, we used the sera from nine GAD65Ab-positive Swedish healthy children. One of the GAD65Ab-positive sera was identified from the group of 85 healthy children recruited from the Stockholm area [21]. The remaining eight healthy sera were identified by screening of sera from 320 Swedish children (0–14 years of age) selected in a previous study [22] as matched control subjects to 494 Swedish IDDM children (GAD65Ab frequency 70 %; A. Falorni unpublished data).

The present study was approved by the ethical committee of the Karolinska Institute.

Rabbit antisera. Rabbit antisera were raised by immunization with synthetic peptides corresponding to peptides specific for human GAD65 or rat GAD67 [23]. The antiserum 10266 was raised with a peptide corresponding to amino acids 2–19 of rat GAD67, 7309 with a peptide corresponding to amino acids 4–22 of human GAD65, 8100 with a peptide corresponding to amino acids 250–269 of human GAD65, and 5482 with a peptide corresponding to amino acids 405–418 of human GAD65.

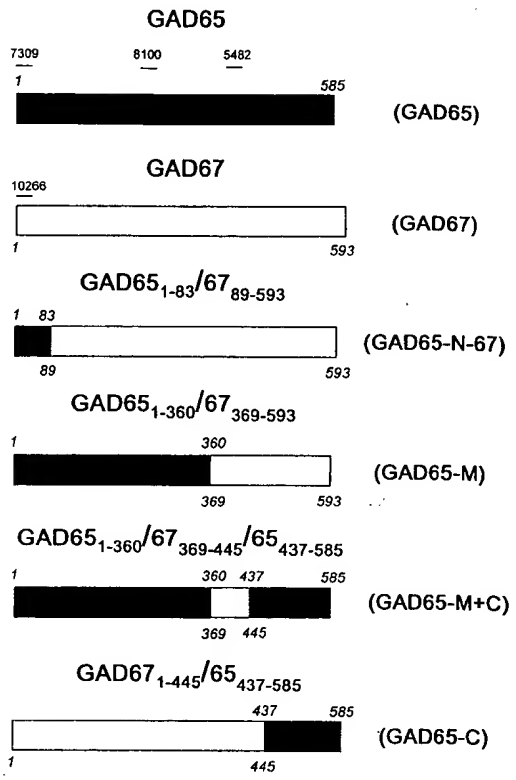

Fig. 1. Schematic representation of the chimaeric molecules used in the study. Numbers are amino acid positions in human GAD65 or rat GAD67. Positions of the peptides recognized by the rabbit antisera used in this study are shown

Construction of chimaeric molecules. Full-length human GAD65 cDNA [1] was inserted into the vector pcDNAII (Invitrogen Corp., San Diego, Calif., USA), and the resultant clone coded pEx9 [8]. Full-length rat GAD67 cDNA [24] was inserted into pGEM4 (Promega, Madison, Wis., USA) and coded pEx12 [8]. The cDNA of rat GAD67 was a kind gift from Dr. B. Michelsen (Hagedorn Research Institute, Gentofte, Denmark). The rat GAD67 isoform shows 98 % amino acid sequence identity to the human GAD67 isoform [25] and is referred to as GAD67.

Figure 1 shows a schematic representation of the chimaeric molecules used in this study. The designations of epitope reactivity of the different molecules are specified in Table 1.

To evaluate the immunoreactivity of the amino-terminal region of human GAD65, we used the DNA coding for a chimaeric molecule containing the amino-terminal region of rat GAD65 (aa 1–83) in fusion with the central/carboxy-terminal regions of rat GAD67 (aa 89–593). The chimaera was coded GAD65$_{1-83}$/67$_{89-593}$. This chimaeric cDNA, a kind gift from Dr. P. De Camilli, Yale University, New Haven, Conn., USA [26], was excised from the original pRC/RSV vector and subcloned into pcDNAII. This molecule detects reactivity against GAD65-N-67 (Table 1).

The GAD65$_{1-360}$/67$_{369-593}$ chimaeric molecule was constructed first by primer extension of a fragment coding for rat

Table 1. GAD chimaeric molecules used to detect GAD65 middle and C-terminal epitopes as well as GAD67 autoantibodies

Chimaeric molecule	Autoantibody primarily detected	Designation
$GAD65_{1-360}/GAD67_{369-593}$	Middle epitope of GAD65	GAD65-M
$GAD67_{1-445}/GAD65_{437-585}$	C-terminal end epitope of GAD65	GAD65-C
$GAD65_{1-360}/GAD67_{369-445}/GAD65_{437-585}$	Middle and C-terminal epitopes of GAD65	GAD65-M + C
$GAD65_{1-83}/GAD67_{89-593}$	GAD67 epitopes and N-terminal GAD65	GAD65-N-67

$GAD67_{368-593}$ in pEx12 using a primer containing a *Bgl* II recognition sequence (AGA TCT) immediately upstream the GAD67 coding sequence, and a primer containing a *Xba* I recognition sequence (TCT AGA) downstream of the stop codon TAA. Then, by taking advantage of the single *Bgl* II site in human GAD65 (base 1073) and of the *Xba* I cloning site in the vector, the *Xba* I-/*Bgl* II-cut amplified $GAD67_{368-593}$ DNA fragment was cloned into the *Xba* I-/*Bgl* II-cut pEx9, downstream of and in-frame with the DNA coding for $GAD65_{1-359}$. This molecule detects antibodies reactive with the middle epitope of GAD65 (GAD65-M; Table 1).

The DNA of a $GAD65_{1-360}/67_{369-445}/65_{437-585}$ chimaera was constructed by taking advantage of a single *Aat* II site (base 1300) in the DNA of $GAD65_{1-360}/67_{369-593}$ chimaera. The DNA fragment coding for $GAD65_{437-585}$ was generated by primer extension of pEx9 using a primer containing an *Aat* II recognition sequence (GAC GTC) immediately upstream of the GAD65 sequence, and a downstream primer containing both a stop codon (TAA) and a *Xba* I recognition sequence. The *Aat* II-/*Xba* I-cut $GAD65_{437-585}$ DNA was ligated into the *Aat* II-/*Xba* I-cut DNA coding for $GAD65_{1-360}/67_{369-593}$. This molecule is used to detect autoantibodies against both the middle and C-terminal end part of GAD65 (GAD65-M + C; Table 1).

To test the immunoreactivity of the carboxy-terminal region of human GAD65, the DNA for a $GAD67_{1-445}/65_{437-585}$ chimaera was constructed using the single *Aat* II site in both rat GAD67 and $GAD65_{1-360}/67_{369-445}/65_{437-585}$, and the single *Sac* I site in both the pcDNAII vector (containing the DNA coding for $GAD65_{1-360}/67_{369-445}/65_{437-585}$) and the pGEM4 vector (containing the DNA coding for GAD67). The *Sac* I-/*Aat* II-cut $GAD67_{1-445}$ DNA was ligated into the *Sac* I-/*Aat* II-cut DNA coding for $GAD65_{437-585}$. It should be noted that $GAD65_{570-585}$ is identical to the homologous region in GAD67 [1, 24, 25]. The $GAD67_{1-445}/65_{437-585}$ chimaera detects autoantibodies against the C-terminal end of GAD65 (GAD65-C; Table 1).

Primer extension reactions were carried out using Taq DNA polymerase (Promega), and the amplified DNA products were purified from a low melting agarose (Gibco BRL, Gaithersburg, Md., USA) gel. All restriction enzymes were from New England Biolabs, Beverly, Mass., USA. DNA ligation was carried out using T4 DNA ligase (Gibco BRL) and an overnight incubation at 16 °C. The ligation buffer was supplemented with ATP at a final concentration of 1 mmol/l. The ligated products were electroporated into competent XL1-blue *Escherichia coli* cells. After transformation, screening of clones was carried out by digestion with selective and informative restriction enzymes. For subsequent DNA sequencing and in vitro transcription/translation, large-scale DNA preparation was performed using a commercially available kit (Qiagen Inc., Chatsworth, Calif., USA). The DNA sequences of all the constructed chimaeric molecules were confirmed by analysis using the Taq DyeDeoxy™ Terminator Cycle Sequencing kit from Applied Biosystems, Foster City, Calif., USA.

In vitro transcription/translation and immunoprecipitation of GAD65, GAD67 and chimaeric molecules. An in vitro coupled transcription/translation system with SP6 RNA polymerase and nuclease treated rabbit reticulocyte lysate (Promega) was used to prepare ^{35}S-labelled GAD and chimaeric molecules following procedures described previously [8, 21]. Efficiency of the in vitro translation was evaluated by determination of the trichloroacetic acid (TCA) precipitable radioactivity [8, 21]. The TCA precipitable radioactivity was: 485000 ± 64000 cpm/µl with $GAD65_{1-83}/67_{89-593}$, 550000 ± 90000 cpm/µl with $GAD65_{1-360}/67_{369-593}$, 510000 ± 87000 cpm/µl with $GAD65_{1-360}/67_{369-445}/65_{437-585}$, 445000 ± 75000 cpm/µl with $GAD67_{1-445}/65_{437-585}$, and 435000 ± 52000 cpm/µl with GAD67, representing 35 ± 4, 39 ± 6, 36 ± 6, 32 ± 5, and 31 ± 4 % of incorporated radioactivity, respectively. These values are similar to those obtained with human GAD65 [21].

The in vitro translated ^{35}S-radiolabelled GAD or hybrid molecules (15000 cpm of TCA precipitable radioactivity in triplicate) were immunoprecipitated overnight with human serum or rabbit antiserum (final dilution 1:25), and the antibody-bound and free antigen separated by protein A-Sepharose using a multiwell-adapted (Millipore Co., Bedford, Mass., USA) procedure described in detail elsewhere [21]. The results were expressed as relative indices using one positive and two negative standard sera previously described [8, 21]. Based on the results of assays (data not shown) with GAD-specific rabbit antisera [23] and the GAD65Ab-positive Juvenile Diabetes Foundation (JDF) world standard for islet cell antibodies (ICA) the following positive standard sera were used in the different assays: the 7309 antiserum in GAD65-N-67 ($GAD65_{1-83}/67_{89-593}$) and GAD65-M ($GAD65_{1-360}/67_{369-593}$), the JDF world standard in GAD65, GAD65-M + C ($GAD65_{1-360}/67_{369-445}/65_{437-585}$) and in GAD65-C ($GAD67_{1-445}/65_{437-585}$) antibody assays, and the 10266 antiserum in the GAD67Ab assay. As negative standards the sera from two healthy individuals [9, 21] were used. Upper levels of normal of the assays were evaluated as mean + 3SD of the indices of the sera from 84 GAD65Ab-negative healthy individuals.

Our GAD65Ab assay participated in the First [27] and Second [28] International Workshops for the Standardization of GAD Antibody Determination and was characterized by the highest sensitivity and specificity. Additionally, in the 1995 Combined Autoantibody Workshop (unpublished data) our GAD65Ab assay had a diagnostic sensitivity of 72 %, as tested in IDDM sera, and a diagnostic specificity of 100 %, as tested in healthy sera.

Statistical analysis

Differences in antibody frequency between IDDM patients and healthy control subjects or between different assays were evaluated using the chi-square test with Yate's correction whenever appropriate or the Fisher's exact test. Correlations among levels of antibodies to the different GAD molecules were analysed by Spearman's rank correlation test after logarithmic transformation of antibody indices. Differences in antibody levels between IDDM and healthy children were tested using the non-parametric Mann-Whitney test. A p value less than 0.05 was considered significant.

Results

The immunoreactivity of the constructed chimaeric molecules was first tested using GAD-specific rabbit antisera and the JDF world standard for ICA (Fig. 2). The sera from two healthy individuals were used as negative controls. The immunoprecipitation of the chimaeric molecules with the rabbit antisera raised by immunization with synthetic peptides of either human GAD65 or rat GAD67 was identical to that predicted to result from the construction strategies.

GAD65Ab and GAD67Ab in IDDM children. GAD65 Ab were found in 116 of 155 (75 %) of our panel of IDDM patient sera [21]. When the samples were analysed for GAD67Ab (upper level of normal: 0.02), 19 of 155 (12 %) were positive, significantly less than GAD65Ab ($\chi^2 = 120.9$, $p < 0.0001$) (Table 2). As many as 17 of 19 (89 %) of the GAD67Ab-positive samples were also GAD65Ab-positive. The levels of GAD67Ab correlated with the levels of GAD65Ab ($r^2 = 0.5913, p = 0.009$) (Fig. 3A) and in 15 of 17 double positive sera the GAD65 index was higher than 0.6 (65th percentile IDDM patients). However, it was noted that only 15 of 34 (44 %) samples with a GAD65 index greater than 0.6 were positive for GAD67Ab.

GAD65-N-terminal Ab in IDDM children. Using an upper level of normal of 0.02, 25 of 155 (16 %) IDDM patients were positive for antibodies to the GAD65-N-67 ($GAD65_{1-83}/67_{89-593}$) chimaera, significantly less than GAD65Ab ($\chi^2 = 105.4$, $p < 0.0001$) but not GAD67Ab ($\chi^2 = 0.662$, p = NS) (Table 2). A total of 23 of 25 (92 %) positive samples were also GAD65Ab-positive and 18 of 25 (72 %) were GAD67 Ab-positive. The two GAD65Ab-negative, GAD65-N-67-Ab-positive samples were also positive for GAD67Ab.

GAD65 middle epitope Ab in IDDM children. The GAD65-M ($GAD65_{1-360}/67_{369-593}$) chimaera reacted similar to GAD65 (Table 2). In particular, using an upper level of normal of 0.035, 104 of 155 (67 %) IDDM patients were positive for middle-epitope Ab,

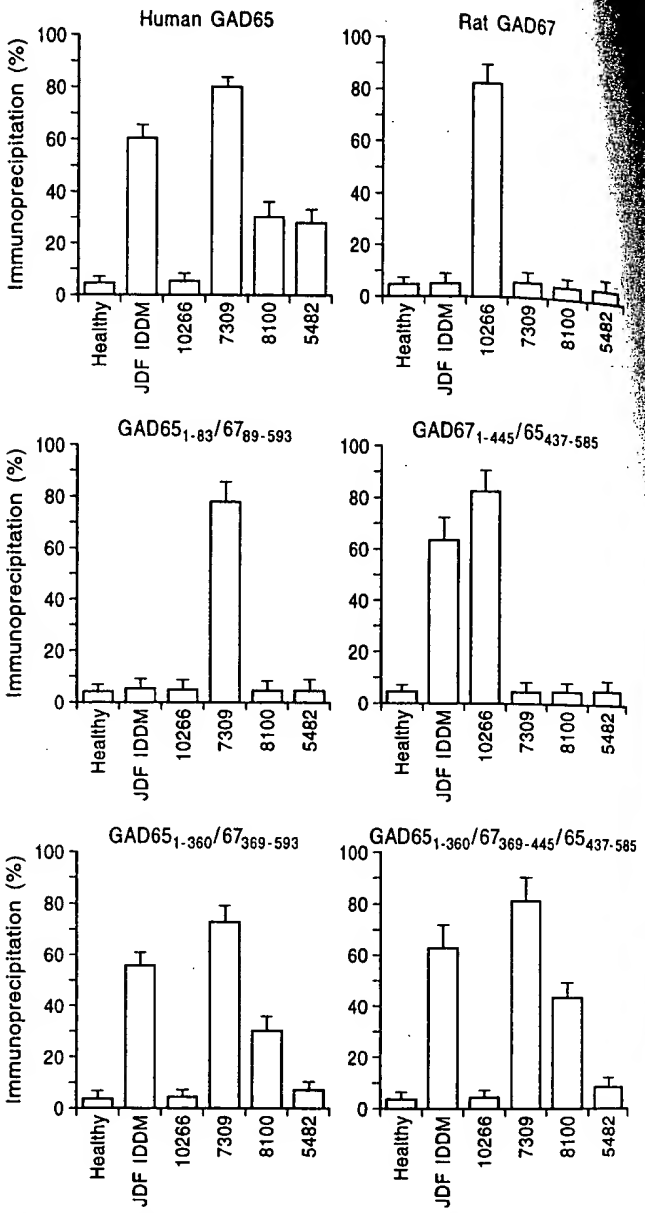

Fig. 2. Immunoprecipitation (%) of human GAD65, rat GAD67 and GAD chimaeric molecules with a healthy human serum, the JDF world standard IDDM serum and four rabbit antisera directed against amino acids 2–19 of rat GAD67 (10266), amino acids 4–22 of human GAD65 (7309), amino acids 250–269 of human GAD65 (8100), and amino acids 405–418 of human GAD65 (5482)

Table 2. Frequency of antibodies to GAD65, GAD67 and GAD chimaeric molecules in IDDM children

^{35}S-GAD antigen[a]	Total frequency	Frequency among GAD65Ab-positive sera	Antibody index[b]
GAD65	116/155 (75 %)	116/116 (100 %)	0.297 (0.033–5.715)
GAD65-C	104/155 (67 %)	104/116 (90 %)	0.422 (0.101–5.061)
GAD65-M	104/155 (67 %)	104/116 (90 %)	0.234 (0.047–3.598)
GAD65-M + C	114/155 (74 %)	114/116 (98 %)	0.355 (0.086–3.915)
GAD65-N-67	25/155 (16 %)	23/116 (20 %)	0.055 (0.022–0.918)
GAD67	19/155 (12 %)	17/116 (15 %)	0.061 (0.023–1.225)

[a] ^{35}S-GAD antigen specified in Table 1;
[b] median (range) in antibody-positive patients

not different from GAD65Ab ($\chi^2 = 1.89$, p = NS) but significantly higher than GAD67Ab ($\chi^2 = 95.1$, $p < 0.0001$). All the GAD65-M-Ab positive samples were also GAD65Ab-positive. The levels of middle-epitope Ab strongly correlated ($r^2 = 0.901$, $p < 0.001$) with the levels of GAD65Ab (Fig. 3B). Only 12 of 116 (10%) GAD65Ab-positive IDDM sera were negative in the middle-epitope Ab assay which suggests that the middle epitope is critical for the IDDM-associated GAD65Ab development.

GAD65 C-terminal end epitope (GAD65-C) Ab in IDDM children. Using an upper level of normal of 0.099, 104 of 155 (67%) IDDM sera were positive for GAD65-C-Ab ($GAD67_{1-445}/65_{437-585}$) (Table 2), not different from GAD65Ab ($\chi^2 = 1.89$, p = NS) but higher than GAD67Ab ($\chi^2 = 95.1$, $p < 0.0001$). All the positive samples were also positive for GAD65 Ab and the levels of GAD65-C-Ab correlated ($r^2 = 0.925$, $p < 0.001$) with GAD65Ab levels (Fig. 3D). As many as 96 of 104 (92%) positive samples were also positive for GAD65-M-Ab. In eight samples, GAD65-C-Ab were found in the absence of GAD65-M-Ab. Similarly, eight GAD65-C-Ab-negative samples were positive for GAD65Ab and for GAD65-M-Ab.

GAD65-M + C-Ab in IDDM children. Using an upper level of normal of 0.085, 116 of 155 (75%) IDDM sera were positive for GAD65-M + C-Ab ($GAD65_{1-360}/67_{369-445}/65_{437-585}$) (Table 2) and the levels correlated ($\chi^2 = 0.95$, $p < 0.001$) with GAD65Ab levels (Fig. 3C).

Analysis of GAD65Ab in IDDM patients. Based on the results of the GAD65Ab and GAD67Ab assays, we identified four groups of IDDM patients (Fig. 4A): 1) GAD65Ab- and GAD67Ab-negative subjects ($n = 37$, 24%), 2) GAD65Ab-positive and GAD67Ab-negative subjects ($n = 99$, 64%), 3) GAD65Ab- and GAD67Ab-positive subjects ($n = 17$, 11%), 4) GAD65Ab-negative and GAD67 Ab-positive subjects ($n = 2$, 1%). The use of a $GAD65_{1-83}/67_{89-593}$ chimaera allowed us to identify 93 of 155 (60%) IDDM patients, representing 80% (93 of 116) of the GAD65Ab-positive subjects, who had antibodies uniquely directed to epitope(s) specific for GAD65 and localized in the $GAD65_{84-585}$ region. Of the remaining GAD65Ab-positive IDDM patients 17 had antibodies against epitope(s) common to both $GAD65_{84-585}$ and $GAD67_{89-593}$. In only 6% (7 of 116) of the GAD65Ab-positive subjects, low levels of antibodies directed to $GAD65_{1-83}$ were detected. These patients were positive for both GAD65Ab and GAD65-N-67-Ab but negative for GAD67Ab. However, it should be noted that in all the 23 GAD65Ab-positive IDDM patients having antibodies to GAD67 or to $GAD65_{1-83}$, high levels

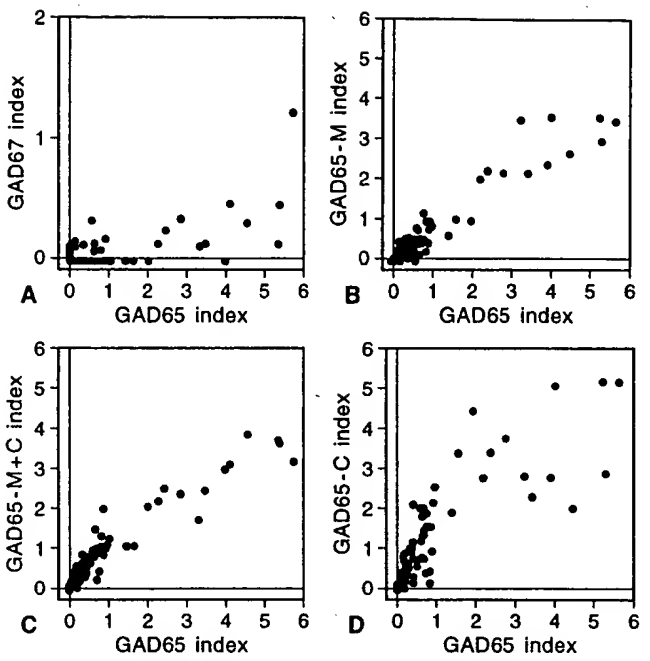

Fig. 3 A–D. Correlations of GAD65Ab levels with **A** GAD67-Ab levels ($r^2 = 0.5913$, $p = 0.009$), **B** GAD65-M-Ab levels ($r^2 = 0.901$, $p < 0.001$), **C** GAD65-M + C-Ab levels ($r^2 = 0.95$, $p < 0.001$), and **D** GAD67-C-Ab levels ($r^2 = 0.925$, $p < 0.001$). Dotted lines show upper levels of normal in each antibody assay

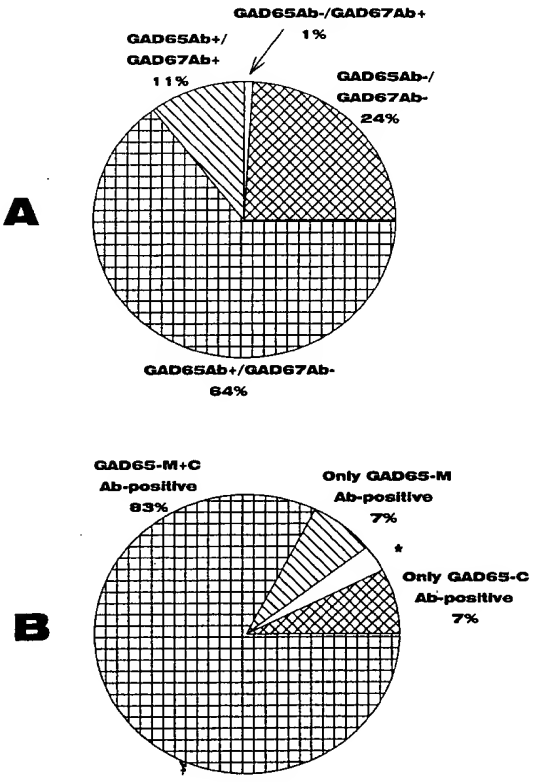

Fig. 4 A, B. Dissection of GADAb in IDDM children. Percentages are frequencies of antibody-positive subjects in the total IDDM population studied (**A**) or in GAD65Ab-positive IDDM children (**B**). *Note that 4 of 116 (3%) GAD65Ab-positive IDDM children were both GAD65-C and GAD65-M antibody negative

Table 3. Frequency of antibodies to GAD65, GAD67 and GAD chimaeric molecules in GAD65Ab-positive healthy children

^{35}S-GAD antigen[a]	Frequency among GAD65Ab-positive sera	Antibody index[b]
GAD65	9/9 (100%)	0.096 (0.044–0.825)
GAD65-C	8/9 (89%)	0.135 (0.106–0.951)
GAD65-M	7/9 (78%)	0.105 (0.052–0.752)
GAD65-M + C	9/9 (100%)	0.122 (0.095–0.798)
GAD65-N-67	2/9 (22%)	0.052 (0.040–0.064)
GAD67	2/9 (22%)	0.067 (0.045–0.089)

[a] ^{35}S-GAD antigen specified in Table 1;
[b] median (range) in antibody-positive patients

of GAD65Ab were detected, suggesting that antibodies to one or more GAD65-specific epitopes localized in $GAD65_{84-585}$ were present also in those patients.

In 96 of 116 (83%) of the GAD65Ab-positive sera, antibodies were found against both the middle and C-terminal end GAD65 epitopes (Fig. 4B). In 8 of 116 (7%) GAD65Ab-positive sera, middle but not C-terminal end Ab were found. Similarly, in 8 of 116 (7%) GAD65Ab-positive sera, C-terminal but not middle Ab were found. Only 4 of 116 (3%) GAD65Ab-positive sera were negative for both middle and C-terminal end Ab.

Frequency of autoantibodies to chimaeric GAD molecules in healthy individuals. Using the same upper levels of normal (see above), none of the 84 GAD65Ab-negative serum samples from healthy Swedish subjects were found to be positive in any of the assays using chimaeric molecules between GAD65 and GAD67. One healthy serum sample was positive for GAD67Ab.

To evaluate whether the GAD65 autoantibody epitopes present in healthy children might be different from those observed in IDDM patients, the sera from nine healthy, GAD65Ab-positive children were tested with GAD67 and all the chimaeric molecules (Table 3). Among the GAD65Ab-positive sera, 2 of 9 (22%) were also GAD67Ab-positive. When tested with the chimaeric molecules, seven of nine (78%) sera were positive for GAD65-M-Ab, nine of nine (100%) sera were positive for GAD65-M + C-Ab, and eight of nine (89%) for GAD65-C-Ab, but only two of nine (22%) were positive for GAD65-N-67-Ab. The two serum samples positive for both GAD65Ab and GAD67Ab were also positive for antibodies to the chimaeric molecules.

The titre of GAD65Ab ($p = 0.007$), GAD65-C-Ab ($p = 0.002$) and GAD65-M + C-Ab ($p = 0.003$), but not of GAD65-M-Ab ($p = 0.101$) was significantly lower in antibody-positive healthy subjects than in antibody-positive IDDM patients.

Discussion

In the present study, we tested 155 IDDM sera against four hybrid molecules constructed by substituting regions of human GAD65 with homologous regions of rat GAD67. We found that: 1) at least two major and distinct GAD65-specific antibody epitopes are localized to the middle (aa 84–360) and carboxy-terminal (aa 437–570) domains of human GAD65 (note that the $GAD65_{570-585}$ sequence is identical to the homologous region in GAD67 [1, 24, 25]; 2) a conservation of GAD65-specific antibody epitope regions characterizes the IDDM patients and 3) titres of carboxy-terminal GAD65 epitope-specific antibodies are significantly higher in IDDM patients than in healthy control subjects and may distinguish IDDM from healthy children.

These results are in agreement with previous reports on GAD65Ab epitopes in IDDM. In a few human GAD-reactive monoclonal antibodies from IDDM patients [16, 29] epitopes were located towards the carboxy-terminal and middle part of the GAD65 molecule [16]. Experiments with deletion mutants and chimaeric molecules between GAD65 and GAD67 confirmed the location of the IDDM-specific epitopes [16, 17, 30, 31]. However, the use of deletion mutants may complicate the interpretation since the IDDM-related GAD65Ab recognise conformational epitopes.

Recently, using 12 IDDM sera and chimaeric molecules between GAD65 and GAD67, Daw and Powers [17] showed the presence of two distinct IDDM-related GAD65Ab epitopes, located to amino acids 240–435 and to amino acids 451–570. Our data confirm and extend these results. We located the two major GAD65-specific epitopes to amino acids 84–360 and 437–570. By combining the regions identified in the two studies, the two major GAD65-specific antibody epitopes can be located to amino acids 240–360 and amino acids 451–570. We demonstrate that the 361–436 region is not critical for IDDM-related autoantibodies since the immunoreactivity of our GAD65-M + C chimaera proved to be indistinguishable from that of GAD65.

Although neither the structure of GAD65 nor that of GAD67 has been determined, it is important to note that most of the GAD65Ab-positive sera were also reactive with the chimaeras used to evaluate the M as well as the C GAD65 epitopes. Since the GAD65 antibodies are conformation-dependent, a loss in antibody reactive structure would have resulted in a major loss of reactivity with the chimaeric molecules. Our data from a large number of GAD65Ab-positive IDDM sera suggest that the use of GAD65/GAD67 chimaeric molecules is successful in localizing conformation-dependent GAD65Ab epitopes. However, further experiments are needed to determine the importance of the GAD65 N-terminal

sequences to influence the conformation of the proposed M epitope. Additionally, as in our study only epitope regions have been detected further studies are needed to determine if each of the two regions can be dissected into multiple epitopes.

In our study we demonstrate the presence of a third distinct GAD antibody specificity directed to an epitope common to both GAD65 and GAD67. In a study on Japanese IDDM patients [11] we showed that GAD67Ab were displaced by an excess of recombinant human GAD65. Similarly, in the present study, most GAD67Ab-positive sera were highly GAD65Ab-positive. However, the fact that only 44% of the high-level GAD65Ab-positive sera (GAD65 index > 0.6) were GAD67Ab-positive demonstrates that the presence of GAD67Ab identifies a subgroup of IDDM patients carrying a distinct antibody specificity. It is noteworthy that Tuomi et al. [32] reported that only a fraction of high-level GAD65Ab-positive IDDM sera reacted with denatured GAD65 by immunoblotting. An intriguing hypothesis is the possibility that sera reacting with denatured GAD have GAD67Ab, probably directed to linear epitopes. Additionally, it should be noted that in patients with other autoimmune diseases, such as Graves' disease, specific GAD67Ab have been detected [33], in the absence of GAD65 Ab. The reason why only a few IDDM patients have GAD65Ab cross-reacting with GAD67 is yet to be clarified.

Our analysis of 155 IDDM sera allowed us to unequivocally demonstrate that the two GAD65-specific antibody specificities are associated in more than 83% of GAD65Ab-positive IDDM patients. The presence of specific antibodies to either the middle or the carboxy-terminal domain of GAD65 is a rare event and may be related to the sensitivity of the antibody assay. The demonstration of high conservation of GAD65Ab epitopes in IDDM patients is of particular interest to our understanding of the molecular mechanisms of autoantibody formation. It is still unclear whether the appearance of GAD65Ab precedes or follows beta-cell destruction. We recently demonstrated [21] that no antibodies to the proinsulin converting enzyme PC-2 (highly expressed in human beta cells) can be found in IDDM patients, suggesting that the autoantibody response does not involve all beta-cell autoantigens. Our data suggest a conserved oligoclonal activation of B-lymphocytes in IDDM, and thereby supports the hypothesis that GAD65Ab formation is strongly related to the pathogenesis of the disease.

In addition, we tested the sera from nine GAD65Ab-positive healthy children not progressing to IDDM in an 8-year follow-up (M. Landin-Olsson, unpublished data). Our study is unique in this regard since no information is available on epitope mapping of GAD65Ab present in healthy individuals.

Although the GAD65 antibody epitopes detected in the nine GAD65Ab-positive healthy subjects were indistinguishable from those observed in the IDDM patients, GAD65-C-Ab index levels were significantly higher in IDDM patients than in healthy control subjects. A high frequency of IDDM-related epitope specificities among currently healthy GAD65Ab-positive children is not unexpected since approximately 10% of GAD65Ab-positive schoolchildren eventually develop IDDM. Our results suggest that progression to IDDM may be associated with an increase in epitope-specific GAD65Ab.

In conclusion, we have shown that IDDM GAD65 Ab are directed to conformational epitopes located in two regions of GAD65, namely $GAD65_{84-360}$ and $GAD65_{437-585}$. Accordingly, the high degree of conservation of GAD65Ab epitope regions among IDDM patients suggests that the epitope-restricted GAD65Ab formation is strongly related to the pathogenesis of the disease, and that progression to IDDM may be associated with an increase in epitope-specific GAD65Ab. Further studies using high resolution epitope mapping are needed to further investigate this possibility.

Acknowledgements. We wish to thank Ms. A. Borch for excellent technical assistance, and Dr. M. Landin-Olsson for follow-up of the healthy children studied in this work. A.Falorni was supported by a Juvenile Diabetes Foundation International Fellowship and by a Visiting Scientist stipend from the Karolinska Institute. The study was supported by the Swedish Medical Research Council (19X-10408), the Novo Nordisk A/S, the Nordisk Insulin Fund, the National Institutes of Health (DK 42654), the Petrus and Augusta Hedlunds Foundation, the Tore Nilsons Fund and Barndiabetesfonden.

References

1. Karlsen AE, Hagopian WA, Grubin CE et al. (1991) Cloning and primary structure of a human islet isoform of glutamic acid decarboxylase from chromosome 10. Proc Natl Acad Sci USA 89: 2115–2119
2. Falorni A, Kockum I, Sanjeevi CB, Lernmark Å (1995) Pathogenesis of insulin-dependent diabetes mellitus. In: Kendall-Taylor P (ed) Autoimmune Endocrine Disease, Bailliere's Clin Endocrinol Metab 9: 25–46
3. Baekkeskov S, Aanstoot H-J, Christgau S et al. (1990) Identification of the 64 K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature (London) 347: 151–156
4. Karlsen AE, Hagopian WA, Petersen JS et al. (1992) Recombinant glutamic acid decarboxylase (representing the single isoform expressed in human islets) detects IDDM-associated 64,000-M_r autoantibodies. Diabetes 41: 1355–1359
5. Velloso LA, Kämpe O, Hallberg A, Christmanson L, Betsholtz C, Karlsson FA (1993) Demonstration of GAD-65 as the main immunogenic isoform of glutamate decarboxylase in type I diabetes and determination of autoantibodies using a radioligand produced by eukaryotic expression. J Clin Invest 91: 2084–2090

6. Seissler J, Amann J, Mauch L et al. (1993) Prevalence of autoantibodies to the 65- and 67-kD isoforms of glutamate decarboxylase in insulin-dependent diabetes mellitus. J Clin Invest 92: 1394–1399
7. Petersen JS, Hejnaes KR, Moody A et al. (1994) Detection of GAD65 antibodies in diabetes and other autoimmune diseases using a simple radioligand assay. Diabetes 43: 459–467
8. Grubin CE, Daniels T, Toivola B et al. (1994) A novel radioligand binding assay to determine diagnostic accuracy of isoform-specific glutamic acid decarboxylase antibodies in childhood IDDM. Diabetologia 37: 344–350
9. Vandewalle CL, Falorni A, Svanholm S et al. (1995) High diagnostic sensitivity of glutamate decarboxylase autoantibodies in insulin-dependent diabetes mellitus with clinical onset between age 20 and 40 years. J Clin Endocrinol Metab 80: 846–851
10. Kawasaki E, Takino H, Mayumi Y et al. (1994) Autoantibodies to glutamic acid decarboxylase in patients with IDDM and autoimmune thyroid disease. Diabetes 43: 80–86
11. Falorni A, Grubin CE, Takei I et al. (1994) Radioimmunoassay detects the frequent occurrence of autoantibodies to the Mr 65, 000 isoform of glutamic acid decarboxylase in Japanese insulin-dependent diabetes. Autoimmunity 19: 113–125
12. Baekkeskov S, Landin-Olsson M, Kristensen JK et al. (1987) Antibodies to a Mr 64, 000 human islet cell antigen precede the clinical onset of insulin-dependent diabetes. J Clin Invest 79: 926–934
13. Bingley PJ, Christie MR, Bonifacio E et al. (1994) Combined analysis of autoantibodies improves prediction of IDDM in islet cell antibody-positive relatives. Diabetes 43: 1304–1310
14. Bingley PJ, Bonifacio E, Gale EAM (1993) Can we really predict IDDM? Diabetes 42: 213–220
15. Hagopian WA, Sanjeevi CB, Kockum I et al. (1995) Glutamate decarboxylase-, insulin-, and islet cell-antibodies and HLA typing to detect diabetes in a general population-based study of Swedish children. J Clin Invest 95: 1505–1511
16. Richter W, Shi Y, Baekkeskov S (1993) Autoreactive epitopes defined by diabetes-associated human monoclonal antibodies are localized in the middle and C-terminal domains of the smaller form of glutamate decarboxylase. Proc Natl Acad Sc. USA 90: 2832–2836
17. Daw K, Powers A (1995) Two distinct glutamic acid decarboxylase autoantibody specificities in IDDM target different epitopes. Diabetes 44: 216–220
18. Björk E, Velloso LA, Kämpe O, Karlsson FA (1994) GAD-autoantibodies in insulin-dependent diabetes mellitus, stiff-man syndrome, and autoimmune polyendocrine syndrome type I recognize different epitopes. Diabetes 43: 161–165
19. Butler MH, Solimena M, Dirkx R, Hayday A, De Camilli P (1993) Identification of a dominant epitope of glutamic acid decarboxylase (GAD-65) recognized by autoantibodies in stiff-man syndrome. J Exp Med 178: 2097–2106
20. Li L, Hagopian WA, Brashear R, Daniels T, Lernmark Å (1994) Identification of autoantibody epitopes of glutamic acid decarboxylase in stiff-man syndrome patients. J Immunology 152: 930–934
21. Falorni A, Örtqvist E, Persson B, Lernmark Å (1995) Radioimmunoassays for glutamic acid decarboxylase (GAD65) and GAD65 autoantibodies using ^{35}S or ^{3}H recombinant human ligands. J Immunol Methods 186: 89–99
22. Landin-Olsson M, Palmer JP, Lernmark Å et al. (1992) Predictive value of islet cell and insulin autoantibodies for type 1 (insulin-dependent) diabetes mellitus in a population-based study of newly-diagnosed diabetic and matched control children. Diabetologia 35: 1068–1073
23. Li L, Jiang J, Hagopian WA et al. (1995) Differential detection of rat islet and brain glutamic acid decarboxylase (GAD) isoforms with sequence-specific peptide antibodies. J Histochem Cytochem 43: 53–59
24. Michelsen B, Petersen JS, Boel E, Møldrup A, Dyrberg T, Madsen OD (1991) Cloning, characterization and autoimmune recognition of rat islet glutamic acid decarboxylase in insulin-dependent diabetes mellitus (IDDM). Proc Natl Acad Sci USA 88: 8754–8758
25. Bu D-F, Erlander MG, Hitz BC et al. (1992) Two human glutamate decarboxylase, 65-kDa GAD and 67-kDa GAD, are each encoded by a single gene. Proc Natl Acad Sci USA 89: 2115–2119
26. Solimena M, Aggujaro D, Muntzel C et al. (1993) Association of GAD-65, but not of GAD-67, with the Golgi complex of transfected Chinese hamster ovary cells mediated by the N-terminal region. Proc Natl Acad Sci USA 90: 3073–3077
27. Schmidli RS, Colman PG, Bonifacio E, Bottazzo GF, Harrison LC, participating laboratories (1994) High levels of concordance between assays for glutamate decarboxylase autoantibodies: the first international glutamic acid decarboxylase antibody workshop. Diabetes 43: 1005–1009
28. Schmidli RS, Colman PG, Bonifacio E, participating laboratories (1995) Disease sensitivity and specificity of 52 assays for glutamic acid decarboxylase antibodies. The second international GADAb workshop. Diabetes 44: 636–640
29. Richter W, Eierman TH, Endl J et al. (1993) Human monoclonal islet specific autoantibodies share features of islet cell and 64 kDa antibodies. Diabetologia 36: 785–790
30. Kim J, Namchuck M, Bugawan T et al. (1994) Higher autoantibody levels and recognition of a linear NH2-terminal epitope in the autoantigen GAD65 distinguish stiff-man syndrome from insulin-dependent diabetes mellitus. J Exp Med 180: 595–606
31. Ujihara N, Daw K, Gianani R, Boel E, Yu L, Powers AC (1994) Identification of glutamic acid decarboxylase autoantibody heterogeneity and epitope regions in type I diabetes. Diabetes 43: 968–975
32. Tuomi T, Rowley MJ, Knowles W et al. (1994) Autoantigenic properties of native and denatured glutamic acid decarboxylase: evidence for a conformational epitope. Clin Immunol Immunopathol 71: 53–59
33. Hallengren B, Falorni A, Landin-Olsson M, Lernmark Å, Papadopoulos KI, Sundkvist G (1996) Islet cell and glutamic acid decarboxylase antibodies in hyperthyroid patients: at diagnosis and following treatment. J Intern Med 239: 63–68